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54 **Novel GTP binding protein and method for production thereof.**

57 The present invention provides a GTP binding protein containing the following amino acid sequence, with a molecular weight of about 22 K dalton and having GTP binding activity which is inhibited by N-ethyl-maleimide and GTP hydrolyzing activity:

Thr-Ile-Glu-Asp-Ser-Tyr, and a method for the production of a GTP binding protein, which comprises introducing a DNA fragment containing DNA that encodes the GTP binding protein into a cloning site present at the downstream to a promoter of an expression vector, then introducing the expression vector thus constructed into a host, culturing said host, thereby expressing and accumulating the GTP binding protein and then collecting thereof.

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## Description

## NOVEL GTP BINDING PROTEIN AND METHOD FOR PRODUCTION THEREOF

## BACKGROUND OF THE INVENTION

## 1. Field of the Invention

The present invention concerns a novel GTP binding protein (Smg p21) which gives an inhibitory effect on ras oncogene products, and a method for producing said protein by utilizing recombinant DNA technology.

## 2. The Prior Art

Each of cells constituting an individual mammal play its own role in such a fashion that the cell always receives extracellular information or stimulation, and responses thereto.

An information conversion unit on a cell membrane for receiving information given to each of cells by so-called primary information-transmitting substance consists of three kinds of proteins, i.e., a receptor, a transducer and an effector. GTP (guanosine-5-triphosphate) binding protein (hereinafter referred to as "G protein") functions as the transducer among them. That is, when the receptor receives primary information from outside of a cell, it acts on an inactive GDP-G protein, by which the GDP bonded to G protein is converted into GTP to form an active GTP-G protein. Then the active GTP-G protein gives an effect on the effector and information (secondary information) is transmitted from the effector to the inside of the cell.

For the G protein, there have been known, for example, functions of high molecular weight G protein (molecular weight of about 40,000) consisting of various proteins (subunits). Recently, the presence of low molecular weight G protein has been revealed.

There are present at least 15 kinds of the low molecular weight G proteins, i.e., G proteins with molecular weight of from 20,000 to 25,000. The present inventors have previously succeeded in purifying G protein with molecular weight of 24,000 (Smg p25A) and G protein with molecular weight of 22,000 (Smg p21 : 22 K dalton) as single protein in SDS-PAGE and reported details for the G protein of Smg p25A (refer to Experimental Medicine, vol. 8, No. 5, p 34 - 42, 1988 : Journal of the Biological Chemistry, 263, p 2897 - 2904, 1988).

However, the structure and function of such G proteins have not yet been revealed in detail.

Furthermore, Smg p21 can be purified only by an amount of about several tens of micrograms by using a conventional methodology in protein chemistry. Accordingly, it has been difficult to use them in a great amount for the pharmaceutical test such as of anti-cancer agents. Further, it has still been considered impossible to introduce artificial mutation, thereby preparing Smg p21 variant protein with a high anti-ras activity.

## SUMMARY OF THE INVENTION

In order to determine or clarify the entire structure and the function of the G protein with molecular weight of 22,000, the present inventors have succeeded for the first time in obtaining the G protein with molecular weight of 22,000 in a great amount as a single molecule species by purifying said G protein, determining its partial amino acid sequence to prepare a probe, cloning cDNA of said G protein from a cDNA library of G proteins, determining the base sequence thereof and constructing an expression vector containing said cDNA.

That is, an object of the present invention lies in providing a GTP binding protein containing the following amino acid sequence, with a molecular weight of about 22 K dalton and having GTP binding activity and GTP hydrolyzing activity in which the GTP binding activity is inhibited by N-ethylmaleimide:

Thr-Ile-Glu-Asp-Ser-Tyr, especially, a GTP binding protein represented by the amino acid sequence shown in Fig. 1.

A further object of the present invention is to provide a method for the production of Smg p21, which comprises introducing a DNA fragment containing DNA that encodes the GTP binding protein into a cloning site present at the downstream to a promoter of an expression vector, then introducing the expression vector thus constructed into a host, culturing the host, thereby expressing and accumulating the GTP binding protein and then collecting thereof.

The present invention is to be described more specifically.

## DETAILED DESCRIPTION OF THE INVENTION

The G protein in accordance with the present invention is present in cytoplasmic membranes of mammalian cells.

For instance, G protein with molecular weight of about 22 K dalton can be purified by pulverizing bovine brain to obtain a fraction of cell membrane, extracting a crude membrane fraction with sodium cholate, and then successively subjecting it to Ultrogel ACA-44 (manufactured by LKB Co.) column chromatography, phenyl-Sepharose CL-4B (manufactured by Pharmacia LKB Co.) column chromatography, hydroxyapatite (manufactured by Seikagaku Kogyo Co.) column chromatography, Mono Q HR 5/5 (manufactured by Pharmacia LKB Co.) column chromatography, Mono S HR 5/5 (manufactured by Pharmacia LKB Co.) column chromatography and then again to Mono Q HR 5/5 column chromatography, as shown in EXAMPLE to be

described later.

The G protein thus purified has GTP binding activity and GTP hydrolyzing activity. Although the GTP binding activity may be inhibited by N-ethylmaleimide, the presence of dithiothreitol would block such inhibition.

Further, the G protein of the present invention shows no cross reaction with an anti-ARF (ADP-ribosylation Factor) polyclonal antibody and an anti-ras p21 monoclonal antibody.

According to the present invention, the partial amino acid sequence of said purified G protein is determined to prepare a probe and CDNA of said G protein is cloned from the CDNA library prepared from the entire poly A RNA by a conventional method.

From the cloned CDNA, a DNA fragment that encodes the G protein with molecular weight of about 22 K dalton is obtained to determine its base sequence (Fig. 3).

It has been found from the base sequence that the G protein according to the present invention is a protein comprising 184 amino acid residues. Further, the G protein according to the present invention has about 77% homology with ras protein for 44 amino acid residues at N-terminals and about 53% homology for the entire portion. And it has been found that they share a region of the same sequence at 35 - 40 (Thr-Ile-Glu-Asp-Ser-Tyr) in which region ras protein is assumed to act with the effector.

The method for the production of the G protein according to the present invention is to be explained.

The DNA fragment which may be used in the present invention includes, in addition to DNA represented in Fig. 3, many derivatives in which a portion of its base sequence may be replaced or deleted or other base may be added in the base sequence, so long as the substance encoded by DNA contained in the DNA fragment has the same physiological activity as Smg p21. For instance, there may be mentioned the DNA that encodes the amino acid sequence shown in Fig. 1 in which the 12th Gly is substituted with Val, the 38th Asp is substituted with Ala and the 40th Tyr is substituted with Lys.

In addition, various amino acid replacements may be included in the amino acid sequence of the G protein according to the present invention, which are summarized in TABLE 1 below.

TABLE 1

Original Residue	Representative Substituent Residue
Ala	Ser
Arg	Lys
Asn	Gln, His
Asp	Glu
Cys	Ser
Gln	Asn
Glu	Asp
Gly	Pro
His	Asn, Gln
Ile	Leu, Val
Leu	Ile, Val
Lys	Arg, Gln, Glu
Met	Leu, Ile
Phe	Met, Leu, Tyr
Ser	Thr
Thr	Ser
Trp	Tyr
Tyr	Trp, Phe
Val	Ile, Leu

The above DNAs which encode the modified G protein according to the present invention may be prepared by a known method such as site-specific mutation using a synthetic oligonucleotide or by a conventional method of ligating a restriction fragment and synthetic one.

The expression vector in the present invention contains a promoter at a suitable position so that it may regulate transcription of the DNA that encodes Smg p21 obtained as described above.

Any promoter may be used so long as it can express Smg p21 in a host, a controllable promoter being further preferred.

For instance, when using as the host microorganisms such as *Escherichia coli* and *Bacillus subtilis*, the expression vector preferably comprises promoter, ribosome binding sequence, Smg p21 gene, transcription termination factor and a gene for controlling the promoter.

As the promoter, there can be mentioned those derived from *Escherichia coli*, phage, etc., for example, triptophan synthetase operon (*trp*), lactose operon (*lac*), lipoprotein (*lpp*), *rec A*, lambda phage, *P<sub>L</sub>*, *P<sub>R</sub>* and T5 early gene *P<sub>25</sub>*, *P<sub>28</sub>* promoters. They may be chemically synthesized. Further, hybrid promoters such as of *tac*

(trp : lac), trc (trp : lac) pac (phage : Escherichia coli), may also be used.

As the ribosome binding sequence, those derived from *Escherichia coli* and phage may be used, but those having consensus sequence synthetically prepared, for example, those having a sequence such as

5 , AGGAGGTTTAA

SD sequence

are preferred.

10 Although the Smg p21 gene may be used as it is, those lacking an unnecessary DNA sequence (non-coding region) by site-specific mutation (BIO TECHNOLOGY, July, p 636 - 639 (1984)) and the like are preferred.

Although the transcription termination factor is not always necessary, it is preferred for the expression vector to have p-independent one, for example, lpp terminator, trp operon terminator, terminator of ribosome RNA gene.

15 Further, usual plasmids may be used for the expression vector of the present invention. Plasmids which may provide a multiple copy in *Escherichia coli* or *Bacillus subtilis*, for example, those derived from pBR 322 or pUB 110 plasmid may preferably be used.

Further, for the sequence order on the expression plasmid, of these factors necessary for the expression, it is desired that they are arranged in the order of promoter, SD sequence, Smg p21 gene, structural gene and transcription termination factor from the upstream at 5' end. There is no particular restriction for the order of the repressor gene required for the control of transcription, marker gene drug resistance gene, etc., initiation site of the plasmid replication and the like. Further, the transcription efficiency can be increased by arranging in tandem and ligating a connected sequence of SD and Smg p21 gene to the downstream of the promoter. As a result, improvement in the yield and quality of the produced protein can be expected.

25 For transformation of the host, usual methods may be used, for example, a method as described in Molecular Cloning, p 250 - 253, (1982) for *Escherichia coli* and methods as described in Molecular General Genetics, 168, p 105 - 115 (1979) and in Proceeding National Academy of Science, U.S.A., 44, p 1072 - 1078 (1958) for *Bacillus subtilis*.

For a method of culturing a transformant, those culture media capable of conducting usual culture both for *Escherichia coli* and *Bacillus subtilis* (Molecular Cloning, p 68 - 73 (1972)) may be used. The culture may be conducted at a temperature from 15 to 42°C and, preferably, within a range causing no induction of the expression of heat shock protein and the like (15 - 25°C).

In eucaryotic cells such as, for example, animal cells, the following conditions are preferred.

Preferred promoters include SV40 early promoter, SV40 late promoter, promoter of apolipoprotein E gene, promoter of apolipoprotein A-I gene, promoter of heat shock gene (Proceeding National Academy of Science, U.S.A., 78, p 7038 - 7042 (1981)), promoter of metallothionein gene (Proceeding National Academy of Science, U.S.A., 77, p 6511 - 6515, (1980)), HSVTK promoter, promoter of adenovirus (Ad 2 major late promoter (Ad 2MLP promoter)), LTR (long Terminal Repeat) of retrovirus, etc., the SV40 promoter and the metallothionein gene promoter being particularly preferred.

40 The expression vector may contain a splice sequence DNA consisting of 5' splice site (5' splice junction donor site), intron and 3' splice site (3' splice junction acceptor site), and intron, in which a common base sequence has been found at exon-intron junction site and the periphery of such junction site, that is, a so-called GT/AG rule has been established that the intron region always begins with 2 base (at donor site) of GT and ends with 2 base (at acceptor site) of AG.

45 One or more of such splice sequence DNA may be present in the expression vector and they may be located either to the upstream or downstream of Smg p21 gene.

As specific examples of the splice sequence DNA, there can be mentioned those splice sequence DNA present in exon 2 and exon 3 of rabbit  $\beta$ -globin gene (Science, 26, p 339 (1979)) and promoter, exon 1, 2 and 3 of metallothionein gene and mouse metallothionein-I gene containing introns A and B (Proceeding, National Academy of Science, U.S.A., 77, 6513, (1980)). Further, 5' and 3' splice sites are not necessarily derived from an identical gene, but a sequence in which 5' splice site contained in adenovirus DNA and 3' splice site derived from Ig variable region gene are ligated together may be alternatively used for instance.

55 The expression vector in the present invention may further contain a polyadenylation site. The polyadenylation site is present downstream of Smg p21 gene. As specific examples of the polyadenylation site, those derived from SV40 DNA,  $\beta$ -globin gene or metallothionein gene may be mentioned. Further, the polyadenylation site may be prepared by ligating polyadenylation sites of  $\beta$ -globin gene and SV40 DNA together.

The expression vector in the present invention may have a dominant selective marker allowing the selection of transformant. If there is no selective marker in the expression vector, the transformed animal cells of the present invention can be selected by cotransformation.

60 As for the selective marker, there can be mentioned DHFR gene giving MTX (methotrexate) resistance, tk gene of herpes simplex virus (HSV) allowing the selection of a transformed tk<sup>-</sup> strain in HAT medium, aminoglycoside 3'-phosphor transferase gene from transposon Tn5 of *Escherichia coli* providing resistance to 3'-deoxystreptomycin antibiotic G418, bovine papilloma virus gene enabling morphological distinction by piled up growth, aprt gene, etc.

For selecting animal cells transformed with the expression vector in the present invention by the cotransformation method, the transformed cells can be selected by transforming animal cells with a plasmid containing the selective marker gene together with the expression vector and selecting the transformed cells based on phenotypes which have appeared by the expression of the selective marker.

It is advantageous if the expression vector contains plasmid fractions having a replication origin derived from cells such as *Escherichia coli*, since it may be cloned in bacteria. pBR 322, pBR 327, pML and the like may be mentioned as examples for such plasmid vector.

As the specific examples of plasmid vectors used for the expression vector in the present invention, there can be mentioned pKCR containing SV40 early promoter, splice sequence DNA derived from rabbit  $\beta$ -globin gene, polyadenylation site from rabbit globin gene, polyadenylation site from SV40 early region and replication origin from pBR 322 and ampicillin resistance gene (Proceeding National Academy of Science, U.S.A., 78, p 1528 (1981)), pKCR H2 in which the pBR322 in pKCR is substituted with pBR327 site and *Eco* RI site present in exon 3 of rabbit  $\beta$ -globin gene is replaced by *Hind*III site (Nature, 307, p 605), pBPVMT1 containing BPV gene and metallothionein gene (Proceeding National Academy of Science, U.S.A., 80, p 398 (1983)), etc.

As animal cells to be transformed with the expression vector, there can be mentioned CHO cells, COS cells, mouse L cells, mouse C127 cells, mouse FM3A cells, etc.

The expression vector of the present invention may be introduced into the animal cells, most generally, by way of transfection, micro injection,  $\text{Ca-PO}_4$  method (Virology, 52, p 456 - 467 (1973)).

Culture of the transformed animal cells may be carried out by a conventional method such as in suspension culture medium or fixed culture medium.

As the culture medium, MEM, RPMI 1640 and the like may be generally used.

The protein thus produced can be separated and purified in the same manner as that used with transformed microorganisms.

As has been described above, since the DNA of the present G protein with molecular weight of about 22 K dalton has extremely high homology (55 %) with an oncogene *ras*, there is a possibility that they may act the same effector. Accordingly, it is considered that the G protein according to the present invention may control an oncogene product protein RAS directly or indirectly, and may be expected to act as an inhibitor against oncogenic effect of RAS.

#### DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates an example of an amino acid sequence for the G protein (Smg p21) according to the present invention.

Fig. 2 shows an elution pattern in Mono Q HR 5/5 column chromatography (the second time) in EXAMPLE 1.

Fig. 3 illustrates a base sequence of the cloned cDNA for the G protein in EXAMPLE 1.

Fig. 4 represents a schema showing the preparation steps of plasmid pSmg 21-1 and pSmg 21-2 in EXAMPLE 2.

Fig. 5 represents a schema showing the preparation steps of plasmid pSmg 21-3 and pSmg 21-4 in EXAMPLE 3.

Fig. 6 represents an outline for the structure of the expression vector pKCR Smg 21 prepared in EXAMPLE 4.

The present invention is to be described more in details referring to EXAMPLES but it should be noted that the present invention is not restricted only thereto unless they does not exceed the scope thereof.

#### EXAMPLE 1

##### (1) Purification of G Protein with Molecular Weight of 22,000 (Smg p21) (refer to Table 2)

(a) Crude G protein with molecular weight (relative molecular weight : Mr) of 20,000 to 25,000 was obtained in accordance with the method of Kikuchi, et al. (Journal of the Biological Chemistry, 263, p 2897 - 2904, 1988).

Specifically, a crude membrane fraction was extracted with sodium cholate from bovine brain, and the fraction was fractionated on Ultrogel AcA-33 column chromatography to separate and obtain a fraction at the second peak among two peaks showing GTP binding activity. The fraction thus obtained was purified on phenyl Sepharose CL-4B column chromatography and then further fractionated on hydroxyapatite column chromatography to separate and obtain a fraction at the first peak. Then, the first peak fraction was fractionated on Mono Q HR 5/5 column chromatography and the fraction at the first peak was separated and collected to obtain a crude G protein with Mr of 20,000 - 25,000.

(b) The crude G protein obtained in (a) above was subjected to Mono S HR 5/5 column chromatography equilibrated with a buffer solution of 50 mM sodium acetate (pH, 5.0) containing 1 mM EDTA, 1 mM dithiothreitol, 5 mM magnesium chloride and 0.6 % CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate). After washing with 5 ml of the same buffer solution, it was eluted out by the same buffer solution (20 ml) with a concentration gradient of sodium chloride (0 - 1.0 M). Elution was carried out at a flow rate of 0.5 ml/min and eluate was fractionated into 0.5 ml each. When the DTP binding activity was examined for each of the fractions, four peaks were observed. Among them, the fourth peak (50 - 68 fractions) was collected, diluted ten times with a buffer solution of 20 mM tris-hydrochloric acid (pH, 8.0) containing 1 mM

EDTA, 1 mM dithiothreitol, 5 mM magnesium chloride and 0.5 % sodium cholate, and subjected to Mono Q HR 5/5 column chromatography equilibrated with the same buffer solution. After washing with 5 ml of the same buffer solution, it was eluted out by the same buffer solution (20 ml) with a concentration gradient of sodium chloride (0 - 0.4 M). Elution was conducted out at a flow rate of 0.5 ml/min and eluate was fractionated each by 0.5 ml. The elution pattern is shown in Fig. 2. When fractions with fraction numbers of 10 - 20 were collected and subjected to SDS-polyacrylamide (8 - 16 %) gel electrophoresis, they showed a single band for about 22 K dalton of molecular weight. Thus, purified G protein (Smg p21) was obtained. Various properties of the G protein (Smg p21) are as shown in TABLE 3.

TABLE 2

	Purification Step	Total Protein	Total GTP Binding Activity	Specific Activity	Yield	
	ml	ml	nmol	nmol/mg	%	
15	Sodium Cholate Extraction	95	858	484	0.56	100
	Ultrogel AcA-44 (second peak)	200	90	223	2.5	46
20	Phenyl Sepharose CL-4B	120	32	142	4.4	29
	Hydroxyapatite (first peak)	240	6.8	51	7.5	11
25	Mono Q HR 5/5 (first peak)	8	0.95	10	11	21
	Mono S HR 5/5 (fourth peak)	9.5	0.23	4.6	20	0.95
30	Mono Q HR 5/5 (second time) (first peak)	5.5	0.011	0.36	33	0.074

TABLE 3

G Protein	
Mr	22,000
Kd of GTP Binding	30 ± 6
Activity (nM)	
GTP Hydrolyzing	0.005 + 0.002
Activity (number of turnovers, min <sup>-1</sup> )	
Effect of N-ethylmaleimide on GTP Binding Activity	Inhibited
Cross Reaction with Anti-ARF Polyclonal Antibody	none
Cross Reaction with Anti-RAS P21 Monoclonal Antibody	none

## (2) Determination of the Amino Acid Sequence of G Protein (Smg p21)

### (a) Preparation of probe

After desalting 25 µg of the purified G protein (Smg p21) obtained in (1) above on Sephadex G-25 chromatography, it was subjected to YMC pack AP-802 C4 column chromatography equilibrated with 0.1 % trifluoroacetic acid. Then, it was eluted out with 40 ml of 0.1 % trifluoroacetic acid having a concentration gradient of acetonitrile/2-propanol (3/7) of from 0 to 100 %, at a flow rate of 1 ml/2 min. The G protein in the resultant fractions was digested with *Achromobacter lyticus* protease I, which was fractionated on Bakerbond

WP Oc-tyl column chromatography. The determination of the amino acid sequence for one of said fractions by a gas phase sequencer (Model 470A, manufactured by Applied Biosystems Co.) has revealed that a peptide contained therein has 18 amino acids as shown below.

Asn-Gly-Gln-Gly-Phe-Ala-Leu-Val-Tyr-Ser-Ile-Thr-Ala-Gln-Ser-Thr-Phe-Asn

Subsequently, an oligonucleotide mixture of the following base sequences was chemically synthesized as a probe based on the sequence : Asn-Gly-Gln-Gly-Phe-Ala (Model 380A, manufactured by Applied Biosystems Co.).

A A C A A  
5'-GCGAAGCCTTGGCCGTT-3'  
C C  
T T

Then, the 5' terminal of the resulting probe was labelled with  $^{32}\text{P}$  by using T4 polynucleotidekinase (manufactured by TOYOBO CO., LTD.) and  $\gamma\text{-}^{32}\text{P}\text{-ATP}$ .

#### (b) Preparation of G protein (Smg p21) cDNA

RNAs having poly(A) were prepared as described below from bovine cerebrum by guanizine thiocyanate-lithium chloride method (Cathala, et al. DNA, 2, p 329, 1983).

Specifically, 5 g of bovine cerebrum was directly frozen in liquid nitrogen, which was then charged together with liquid nitrogen into a whirling blender and pulverized for 2 min at 3,000 rpm. The resulting powder was further pulverized and solubilized in 100 ml of a solution comprising 5 M guanizine thiocyanate, 10 mM EDTA, 50 mM tris-hydrochloric acid (pH 7) and 8 % (V/V)  $\beta$ -mercaptoethanol, using a teflon homogenizer (5 rpm). 20 ml of the solubilized material was quietly placed on 10 ml of a 57 M cesium chloride solution in a centrifugal tube, centrifuged at 27,000 rpm for 20 hours by Hitachi RPS28-2 rotor and then RNAs were recovered as precipitates. The RNA precipitates were dissolved in 10 ml of a solution comprising 0.1 % sodium lauryl sulfate, 1 mM EDTA and 10 mM tris-hydrochloric acid (pH 7.5), extracted with phenol-chloroform and then recovered by ethanol precipitation. About 3.95 mg of the resultant RNA was dissolved in 1 ml of a solution comprising 10 mM of tris-hydrochloric acid (pH 8.0) and 1 mM EDTA, incubated at 65°C for 5 min and mixed with 0.1 ml of 5 M sodium chloride. The mixture was subjected to oligo (dT) cellulose-column chromatography (column volume of 0.5 ml) (manufactured by P-L Biochemical Co.).

mRNA having an adsorbed poly (A) was eluted out with a solution comprising 10 mM tris-hydrochloric acid (pH, 7.5) and 1 mM EDTA to obtain about 100  $\mu\text{g}$  of mRNA having poly (A).

By using about 5  $\mu\text{g}$  of the poly(A) mRNA thus obtained, a library of bovine brain cDNA ( $\lambda\text{gt} 10$  vector) was prepared in accordance with the method as described in a manual of Amersham Co. "cDNA Synthesis System", p 13 -21 and "cDNA Cloning System  $\lambda\text{gt} 10$ ", p 11 - 28. From the resulting cDNA library, a plaque hybridized with the labelled probe obtained in (a) above on a nitrocellulose filter (manufactured by S & S Co.) was detected by the method of D Hanahan, et al (Methods in Enzymology, 100, p333 - 342, 1983) to obtain 1 clone of  $\lambda\text{gt} 10$  hage integrated with the cDNA coding for the G protein (Smg p21).

The resulting phage DNA was digested with *Eco* RI to obtain about 2 Kb of *Eco* RI fragment, which was inserted into an *Eco* RI site of plasmid pUC 19 (Gene, 33,34, p 103 -119, 1985) to clone DNA that encodes the G protein (Smg p21). The resultant plasmid was defined as pSmg 21. The base sequence of the thus obtained DNA was determined by the dideoxy method of Sanger, et al (Proceeding of the National Academy of Science, U.S.A., 74, p 5463 - 5467, 1977). The results are shown in Fig. 3.

From the base sequence described above, it was found that the G protein (Smg p21) the entire amino acid sequence as shown in Fig. 1.

#### EXAMPLE 2

##### A : Preparation of Expression Vector and Transformant

##### 1) Mutation at N terminal

(1) pSmg 21 was treated by the method as described in the catalogue of Takara Shuzo Co. in 1988 (p 82 and 83) to obtain 10  $\mu\text{g}$  of a single strand DNA.

(2) A primer of the following portion to be used for mutation was synthesized in a DNA synthesizer (Applied Biosystem Model 380A, manufactured by Nikkaki Co.). The synthesized DNA was reacted with a concentrated aqueous ammonia at 65°C over one night to remove a protective group and purified by a reverse-phase HPLC. Primer :

5'-CGGCCAGTGAATTCC

AAGCTTATGAGAGAATATAAAGTAGT

GGTCCCTTGG-3'

150 pmol of the primer was phosphorylated at 5' end with 20 units of T4 polynucleotide kinase in a system comprising 10 µl of a kinase buffer solution (50 mM of tris-HCl (pH, 8.0), 10 mM magnesium chloride and 5 mM dithiothreitol).

(3) Then, a mutant having a double strand DNA was prepared from 8 pmol of the 5'-phosphorylated primer in (2) and 10 µg of the single strand DNA obtained in (1) above in a system for preparing a mutant with site-specific mutation in vitro using oligonucleotide of Amersham Co., in accordance with an manual of Amersham Co. (p 25 - 32, 1988).

By using 2 µl of an aqueous solution containing the resulting circular DNA, *Escherichia coli* HB 101 strain was transformed in accordance with a customary method to obtain a transformant. The plasmid was separated and purified from the transformant by a customary method, which was then cleaved by a restriction enzyme HindIII to obtain two fragments as a mutant plasmid by means of 5 % acrylamide gel electrophoresis. In this way, plasmid pSmg 21 - 1 was obtained.

## 2) Introduction of Smg p21 cDNA into Expression Vector.

(1) 10 µg (-3 pmol) of pSmg 21 - 1 was cleaved by using 20 units of HindIII and 20 units of Bgl II at 37°C for 2 hours in 100 µl of a buffer solution H (10 mM tris-hydrochloric acid (pH, 7.5), 100 mM sodium chloride and 6 mM magnesium chloride). The cleaved plasmid was subjected to 5 % acrylamide gel electrophoresis to separate and purify an about 700 bp DNA fragment that encodes Smg p21. -----Fragment N.

(2) 2 µg of pUS ΔH (-1 pmol) as an expression vector was cleaved using 2 units of HindIII and 2 units of Bgl II in a 20 µl of the buffer solution H at 37°C for 2 hrs. The reaction product was extracted and deproteinized with water-saturated phenol of an equivalent volume. After extracting phenol with ether, the extracted product was dialyzed against, to water, desalted and then concentrated by a vacuum pump to obtain 10 µl of an aqueous solution containing an expression vector fragment HB.

(3) 0.5 pmol of Fragment N and 0.1 pmol of the expression vector fragment HB were mixed and treated with 1 unit of T4 DNA ligase at 4°C in 10 µl of a buffer solution comprising 10 mM of tris-hydrochloric acid (pH, 7.5), 1 mM dithiothreitol, 8 mM magnesium chloride and 1 mM ATP. 3 µl of the resulting vector was used for transforming commercially available *Escherichia coli* JM 109 competent cells by a conventional method. The transformant was selected in L-culture medium containing 20 µg/ml of ampicillin (10 g/l of bactopeptone, 5 g/l of yeast extract, 10 g/l of sodium chloride and 15 g/l of agar) to obtain the expression vector pSmg 21-1 in which a gene encoding a specific antigen had been inserted (Fig. 4).

## B : Expression of Smg p21

*Escherichia coli* YA21 strain carrying pSmg 21-2 was cultured in L-broth at 30°C over one night. Then, it was inoculated to M9 culture medium (adjusted to pH 7.4 with 6 g/l of disodium hydrogenphosphate, 3 g/l of sodium dihydrogenphosphate, 0.5 g/l of sodium chloride and 1 g/l of ammonium chloride and then supplemented with 2 ml of 1 M magnesium sulfate, 10 ml of 20% glycerol and 0.1 ml of 1M calcium chloride) so as to be diluted 50 times and cultured under shaking at 30°C for 2 hours. Then, IPTG (Isopropyl-β-D-galactopyranoside) was added to the culture medium to a final concentration of 2 mM. After incubated under shaking at 30°C for 16 hours, bacteria were collected by centrifugation at 6,500 rpm for 10 min. They were suspended and maintained in a buffer solution comprising 0.9 % sodium chloride and 10 mM tris-hydrochloric acid (pH, 7.5).

## C : Confirmation of the Expression of Smg p21

After subjecting 0.3 ml of the cultured cell bodies obtained in (B) above to 10 % SDS polyacrylamide gel electrophoresis (in a buffer solution comprising 3 g/l of tris, 14.4 g/l of glycine and 0.1 % of SDS, at 120 V for one hour), the gel was taken out and dyed with Coomassie Brilliant Blue (Sigma Co.) by an usual method. As a result, a new band which was not found for cell bodies cultured without induction of IPTG was detected at the region for a molecular weight of about 22,000, which was expected to be Smg p21. Then, the gel corresponding to the new band was cut-out and again subjected to electrophoresis in the same buffer solution to elute out a protein into the buffer solution. When an amino acid sequence for the eluted protein was determined from the N-terminal by an amino acid sequencer (manufactured by Applied Bio-Systems Co.), it was revealed that the protein was identical to Smg p21 as far as N-terminal 20 amino-acid sequence.

Further, when the resultant protein was reacted with an anti-Smg p21 mouse monoclonal antibody on a nitrocellulose filter, washed, labelled with Iodo-labelled protein A, it was found that the protein may react with the anti-Smg p21 mouse monoclonal antibody.

From the above results, it was confirmed that the resultant protein was Smg p21.



## EXAMPLE 3

10 µg of the plasmid pSmg 21-2 obtained in EXAMPLE 2 was cleaved with each 10 units of Bam HI and Bgl II, while being kept together at a temperature of 37°C for 2 hours.

The reaction product was subjected to 4 % polyacrylamide gel electrophoresis and a Bam HI and Bgl II fragment of about 700 bp was cut-out from the gel and purified. Separately, 10 µg of plasmid pSmg 21-2 was cleaved with 10 units of Bam HI while being kept at a temperature of 37°C for 2 hours and, thereafter, maintained at 65°C for one hour together with 2 units of alkaline phosphatase to remove a terminal phosphoric group. Then, phenol extraction and ethanol precipitation were conducted for purification. 0.1 µg of the plasmid thus purified was ligated with the above Bam HI and Bgl II fragment by using T4 ligase. Among the various resultant plasmids, plasmids pSmg 21-3 and 21-4 having two Smg p21 genes or more were obtained (Fig. 5).

Escherichia coli YA 21 strain was transformed with pSmg 21-3 and pSmg 21-4 respectively to obtain transformed Escherichia coli strains producing Smg p21.

After culturing these Escherichia coli strains at 30°C for one day in 10 ml of LB broth (10 g/l of bactotryptone, 5 g/l of bactoyeast extract and 10 g/l of sodium chloride : pH, 7.5), they were inoculated to 1 liter of M9 culture medium and cultured under shaking at 19°C for 2 hours. Then, IPTG was added to a final concentration of 2 mM and further cultured under shaking at 19°C over one day and night, by which Smg p21 recovered in soluble fractions could be produced. The soluble fraction means herein a fraction prepared by collecting bacteria after culture, and subjecting them serially lysozyme treatment at a lysozyme concentration of 10 mg/l, supersonic pulverization for 2 min using a Sonicator (manufactured by Bulanson Co.) and then centrifugation to the thus obtained cell bodies in a solution at a final concentration of 0.1 % triton x 100 and 1.5 M of sodium chloride at 15,000 rpm for 10 min and then recovering a resulting supernatant. When the activity of the soluble fraction with the anti-Smg p21 mouse monoclonal antibody was examined in the same manner as in EXAMPLE 1, it was found that the fraction contained protein capable of binding with said antibody.

## EXAMPLE 4

## A : Preparation of Expression Vector

After digesting 10 µg of pSmg 21 with 10 units of Eco RI, it was separated by 0.7% agarose gel electrophoresis and agarose gel portion containing an about 2.5 Kbp DNA fragment was cut out and DNA was extracted from the gel by a freeze-thawing method.

The extracted DNA was purified by repeating phenol extraction and ethanol precipitation.

0.5 µg of the DNA was reacted with 0.1 µg of a pKCR vector previously digested with Eco RI and treated with alkaline phosphatase treatment (Japanese Patent Application Laid-Open (KOKAI) No. 285990/86) in the presence of 5 units of T4 DNA ligase at 16°C over one day and night to obtain an expression vector pKCR Smg 21, which is shown in Fig. 6.

## B : Expression of Smg p21 in COS Cells

20 µg of pKCR Smg 21 obtained in (A) above was added to a dish (8 cm in diameter) containing COS cells (about  $\times 10^6$  cells) cultured in a semi-confluent state and introduced into the COS cells by a calcium phosphate method in accordance with the method of Okayama, et al. ("Molecular and Cellular Biology", 17, p 2745 - 2752, 1987). After introduction, they were cultured in CO<sub>2</sub> atmosphere at 37°C over three days and nights to obtain about  $2 \times 10^7$  cells.

From the cultured cells thus obtained, total RNA (about 100 µg) was obtained by a GTC-lithium chloride method ("Molecular cloning, first addition", p 188 - 196, 1982, published from Cold Spring Harbor Laboratory).

Using 20 µg of the RNA, Northern blotting was applied in accordance with the method of Tomas, et al ("Methods in Enzymology", 100, p 255 - 266, 1983, published from Academic Press). In this, case, an about 660 bp DNA fragment obtained by digesting 1 µg of pSmg 21 with one unit of Eco RI and one unit of Bgl II (Smg p21 coding probe, having all of the Smg p21 coding region therein and labelled with <sup>32</sup>P by nick translation was used as a probe.

For comparison, COS cells not introduced with pKCR Smg 21 were cultured in the same way and the Northern blotting was applied by using the total RNA prepared in the same manner as above from the cultured cells thus obtained.

As a result, in the RNA obtained from the COS cells bearing pKCR Smg 21, there was detected RNA to be hybridized with the Smg p21 coding probe in the extra region longer than about 2 Kpb, which was not detected for the RNA obtained from COS cells not transformed with the vector.

Accordingly, it was confirmed that the Smg p21 gene was expressed from pKCR Smg 21.

## EXAMPLE 5

A mutant DNA of Smg p21 gene, in which a base sequence coding for Gly (12) is replaced with that coding for Val(12), was prepared and expressed in host cells.

(1) A DNA fragment having the following base sequence:

5'-CTAGTGGTCCTTGGATCCGTAGGCGTGGGGAAG-3'

was prepared by using a DNA synthesizer (manufactured by Applied Bio-Systems Co.). The resulting oligomer (50 pmol) was dissolved in 50 µl of a solution containing 100 mM tris-hydrochloric acid (pH, 8.0), 10 mM magnesium chloride, 7 mM dithiothreitol and 1 mM ATP, treated with 2 units of T4 polynucleotide kinase at

5

10

15

20

25

32

95

45

50

55

10

101	105	110	
Leu-Arg-Val-Lys-Asp-Thr-Glu-Asp-Val-Pro-			
111	115	120	5
Met-Ile-Leu-Val-Gly-Asn-Lys-Cys-Asp-Leu-			
121	125	130	10
Glu-Asp-Glu-Arg-Val-Val-Gly-Lys-Glu-Gln-			
131	135	140	
Gly-Gln-Asn-Leu-Ala-Arg-Gln-Trp-Cys-Asn-			
141	145	150	15
Cys-Ala-Phe-Leu-Glu-Ser-Ser-Ala-Lys-Ser-			
151	155	160	20
Lys-Ile-Asn-Val-Asn-Glu-Ile-Phe-Tyr-Asp-			
161	165	170	
Leu-Val-Arg-Gln-Ile-Asn-Arg-Lys-Thr-Pro-			
171	175	180	25
Val-Glu-Lys-Lys-Lys-Pro-Lys-Lys-Lys-Ser-			
181	184		30
Cys-Leu-Leu-Leu			

(3) A method for the production of a GTP binding protein, which comprises introducing a DNA fragment containing DNA that encodes the GTP binding protein as defined in Claim 2 into a cloning site present at the downstream to a promoter of an expression vector, then introducing the expression vector thus constructed into a host, culturing said host, thereby expressing and accumulating the GTP binding protein and then collecting thereof.

Fig.1(i)

1 5 10  
 Met-Arg-Glu-Tyr-Lys-Leu-Val-Val-Leu-Gly-  
 11 15 20  
 Ser-Gly-Gly-Val-Gly-Lys-Ser-Ala-Leu-Thr-  
 21 25 30  
 Val-Gln-Phe-Val-Gln-Gly-Ile-Phe-Val-Glu-  
 31 35 40  
 Lys-Tyr-Asp-Pro-Thr-Ile-Glu-Asp-Ser-Tyr-  
 41 45 50  
 Arg-Lys-Gln-Val-Glu-Val-Asp-Cys-Gln-Gln-  
 51 55 60  
 Cys-Met-Leu-Glu-Ile-Leu-Asp-Thr-Ala-Gly-  
 61 65 70  
 Thr-Glu-Gln-Phe-Thr-Ala-Met-Arg-Asp-Leu-  
 71 75 80  
 Tyr-Met-Lys-Asn-Gly-Gln-Gly-Phe-Ala-Leu-  
 81 85 90  
 Val-Tyr-Ser-Ile-Thr-Ala-Gln-Ser-Thr-Phe-  
 91 95 100  
 Asn-Asp-Leu-Gln-Asp-Leu-Arg-Glu-Gln-Ile-  
 101 105 110  
 Leu-Arg-Val-Lys-Asp-Thr-Glu-Asp-Val-Pro-  
 111 115 120  
 Met-Ile-Leu-Val-Gly-Asn-Lys-Cys-Asp-Leu-

*Fig.1(ii)*

121                      125                      130  
Glu-Asp-Glu-Arg-Val-Val-Gly-Lys-Glu-Gln-

131                      135                      140  
Gly-Gln-Asn-Leu-Ala-Arg-Gln-Trp-Cys-Asn-

141                      145                      150  
Cys-Ala-Phe-Leu-Glu-Ser-Ser-Ala-Lys-Ser-

151                      155                      160  
Lys-Ile-Asn-Val-Asn-Glu-Ile-Phe-Tyr-Asp-

161                      165                      170  
Leu-Val-Arg-Gln-Ile-Asn-Arg-Lys-Thr-Pro-

171                      175                      180  
Val-Glu-Lys-Lys-Lys-Pro-Lys-Lys-Lys-Ser-

181                      184  
Cys-Leu-Leu-Leu

Fig. 2

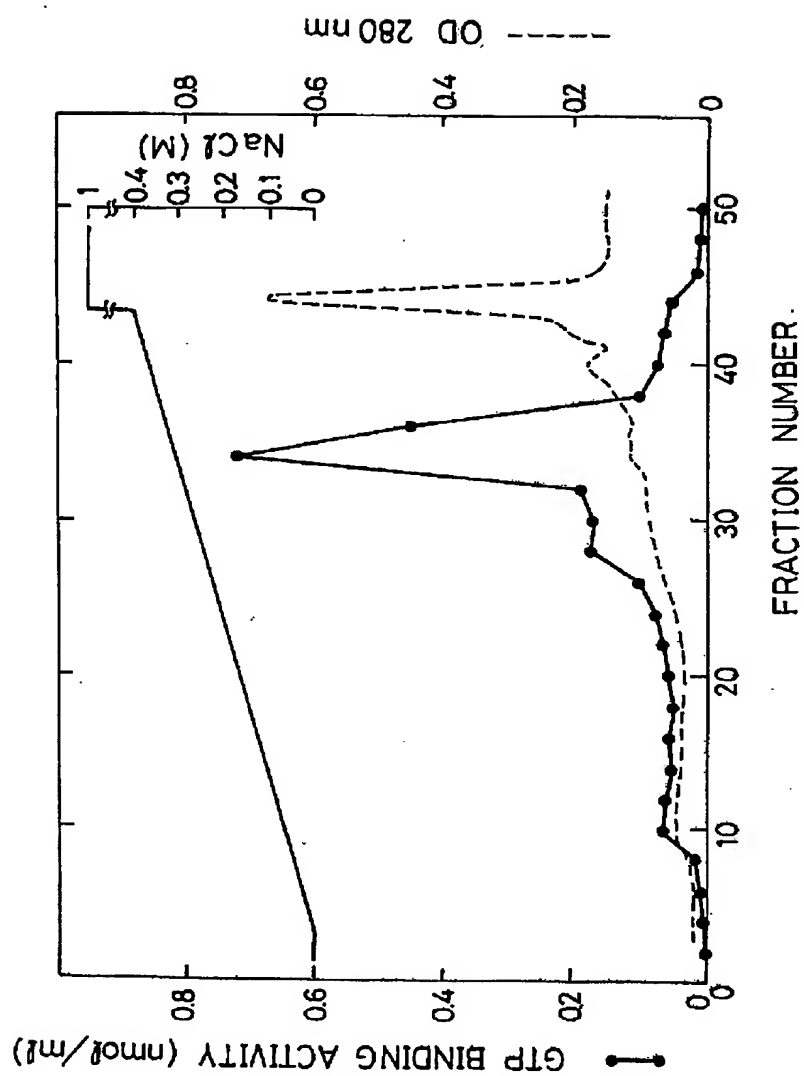


Fig. 3

CCACATCAIGCGTGAGTACAAGCTAGTGGTCCTTGGTTCAGGAGGCGTGGGGAAGTCTGC  
TCTGACAGTTCAGTTTGTTCAGGGAATTTTGTGAAAAATATGACCCACGATAGAAGA  
TTCCTACAGAAAGCAAGTTGAAGTAGACTGCCAACAGTGATGCTCGAAATCCTGGACAC  
AGCGGGAACAGAGCAATTTACAGCAATGAGGGATTTGTATATGAAGAATGCCAAGGGTT  
TGCAC TAGTATATTCTATTACCGCTCAGTCCACATTTAATGACTTACAAGACCTGAGGGA  
ACAGATTTTACGAGTTAAGGACACAGAGATGTTCCAATGATTTTGGTTGGCAATAAGTG  
TGACCTGGAAGACGAGGAGTAGTTGGCAAAGAACAGGGTCAGAAATTTAGCAAGACAGTG  
GTGTAAC TGTGCCCTTTTTTAGAATCTTCTGCAAAGTCAAAGATCAACGTTAATGAGATATT  
TTATGACCTGGTCAGACAGATAAATAGAAAAACACCAGTGGAAAAAGAGAAAGCCTAAAAA  
GAAATCGTGTCTGCTGCTTTAGACCCACAGTAAGCAGCAGCTCTGAGCCAGATTACAGGA  
ATGAAGAACTGTTGCCTAATTGGAAAGTGCCAGCATTCCATACTTCAAAAAATAAATCTGA  
AGAGGCTTCTCCTGTTTTATATATTATGTGAAGAAATTTAGATCTTATATTGGTTTGCACA  
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TCTCCTTTGCAATAGCAGTTATAA

Fig. 4

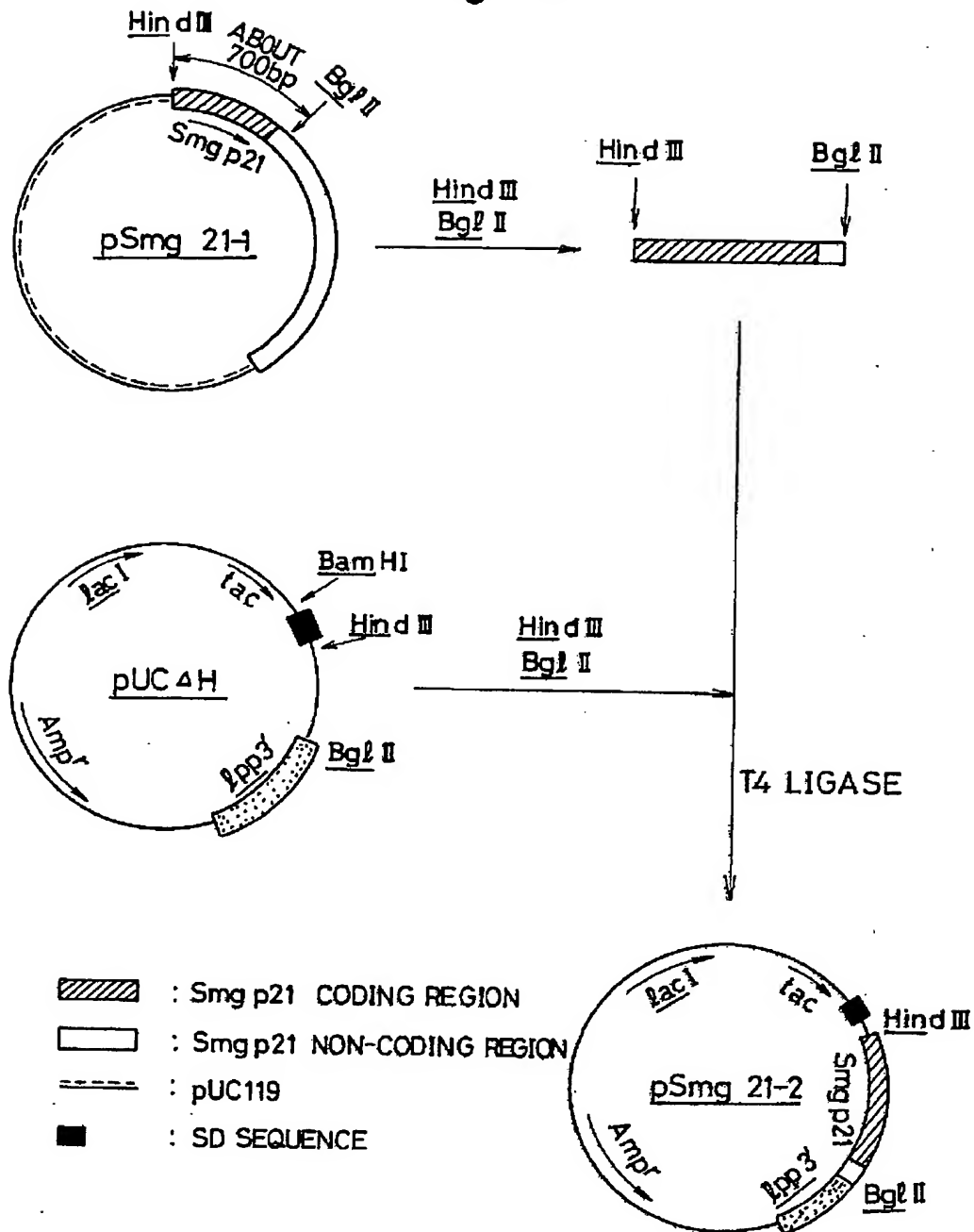




Fig. 5

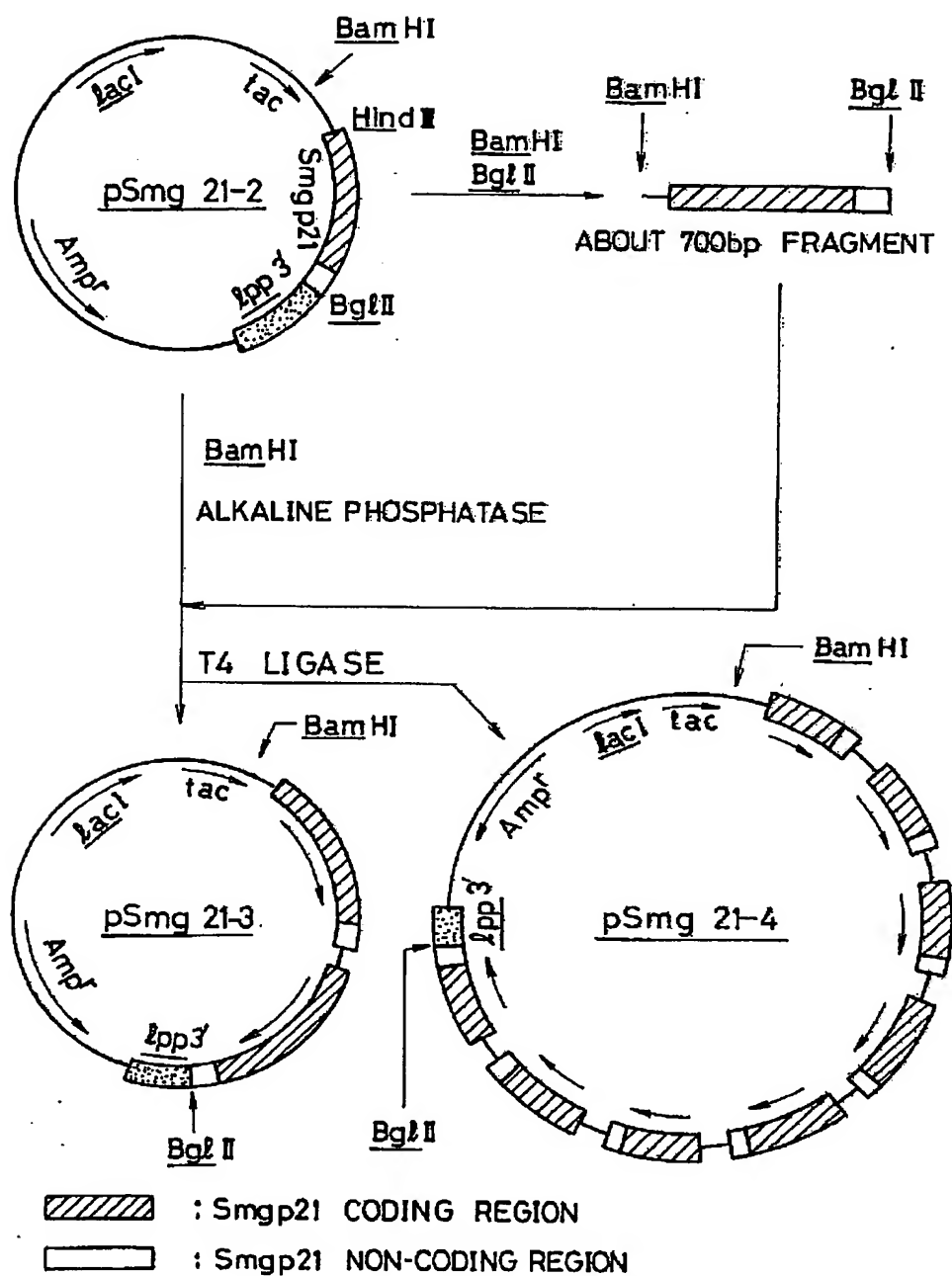


Fig.6

